Oxygen-Induced Membrane Depolarizations in Legume Root Nodules

Possible Evidence for an Osmoelectrical Mechanism Controlling Nodule Gas Permeability

R. Ford Denison[1]* and Thomas B. Kinraide

Appalachian Soil and Water Conservation Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Beckley, West Virginia 25802

Various stresses trigger rapid and reversible decreases in the O_2 permeability (P_O) of legume root nodules. Several possible mechanisms have been proposed, but no supporting data have previously been presented that meet the requirements for both rapidity and reversibility. Stomatal regulation of gas permeability in leaves involves electrically driven fluxes of inorganic osmoticants, so we investigated the possibility of a somewhat similar mechanism in nodules. We used microelectrodes to monitor membrane potential in intact, attached nodules of *Glycine max*, *Medicago sativa*, *Lotus corniculatus*, and *Trifolium repens* while controlling external O_2 concentration and, in the case of *G. max*, measuring P_O with a nodule oximeter. A 1- to 2-min exposure to 100 kPa O_2 was found to induce rapid and reversible membrane depolarizations in nodules of each species. This depolarization (which, to our knowledge, is unique to nodules) is accompanied by reversible decreases in P_O in *G. max* nodules. An osmoelectrical mechanism for control of nodule gas permeability, consistent with these data, is presented.

Legume root nodules must maintain low O_i to protect nitrogenase from O_2 inactivation while still allowing high O_2 flux to support the respiratory requirements of N_2-fixing bacteroids. O_2-related adaptations of nodules include a gas diffusion barrier in the inner cortex (Tjepkema and Yocum, 1974) and Lb-facilitated diffusion of O_2 within infected cells (Appleby, 1984).

Nodule gas permeability is under physiological control. A variety of stresses, including drought (Weisz et al., 1985), defoliation (Hartwig et al., 1987), nitrate (Schuller et al., 1988), and elevated external O_2 concentration (King et al., 1988) cause decreased permeability to O_2 and other gases. Decreased P_O may protect nitrogenase from O_2 inactivation when stress limits respiratory capacity to consume O_2 diffusing into the nodule central zone. Decreases in P_O can occur within minutes and are normally reversible, at least in response to changes in external O_2 (Denison and Layzell, 1991).

It is widely assumed that decreases in P_O caused by different stresses involve the same physical mechanism, although possibly different signal transduction pathways. Several possible mechanisms have been proposed, including (a) changes in intercellular air spaces due to the synthesis or movement of organic (Layzell et al., 1990; Streeter, 1992; Purcell and Sinclair, 1994) or inorganic (Witty and Minchin, 1990) osmoticants, (b) changes in the concentration or O_2 affinity of Lb (Hunt and Layzell, 1993), and (c) deposition of a glycoprotein in intercellular spaces (James et al., 1991; De Lorenzo et al., 1993; Iannetta et al., 1993). The glycoprotein hypothesis is supported by the most published experimental data, but none of these publications has yet shown that glycoprotein deposition is either rapid or reversible.

A possible osmoelectrical mechanism for the control of P_O is considered here. The proposed mechanism shares some features with the processes that control stomatal conductance and pulvinar movements. Under this hypothesis, decreased P_O would result from efflux of inorganic ions and water from nodule cells, accompanied by membrane depolarizations. As a preliminary test of this hypothesis, E_m was monitored in nodules of four legume species during O_2-induced changes in P_O.

MATERIALS AND METHODS

Growth of Plants

Birdsfoot trefoil (*Lotus corniculatus* cv Fergus), alfalfa (*Medicago sativa* cv Weevlchek), white clover (*Trifolium repens* cv Huia), and soybean (*Glycine max* cv Williams) plants inoculated with rhizobial strains 95C11, 102F77B, 162S7A, and USDA 110, respectively, were grown in growth pouches under controlled conditions as previously described (Denison and Layzell, 1991), except that the nitrogen-free nutrient solution of Vessey and Layzell (1987) was modified by substituting Fe-N,N'-ethylenebis-2,2'-hydroxyphenylglycine (Chaney and Bell, 1987) for Fe-Sequestrene 330. The number of plants per pouch was one for soybean and three for the other species.

[1] Present address: Department of Agronomy and Range Science, University of California, Davis, CA 95616.

* Corresponding author; e-mail rfdenison@ucdavis.edu; fax 1–916–752–4361.

E_m Measurements

The E_m of nodule cells was measured with glass micropipette electrodes filled with 2 M or 10 mM KCl using standard electrophysiological circuitry. The nodules remained attached to whole seedlings reared in growth pouches, and care was taken to reduce disturbance of the plant. To gain access to a nodule, a rectangle of plastic and paper that covered one side of the nodule was cut away, leaving a nodule exposed on one side and covered with clear plastic on the other side. The pouch was laid on the stage of an inverted microscope with the exposed side of the nodule up and the plastic-covered side down (Fig. 1). Water, nutrient solution, or 10 mM KCl was dropped onto the nodule. The solution quickly wicked away from the nodule along the attached root and into the nearby paper, but a visible layer of solution remained on at least part of the nodule surface. This layer is essential; whenever it dried, the E_m was either difficult to measure or became less negative than expected for healthy plant tissue, which is normally more negative than -80 mV. As soon as the pouch was positioned, a gas-delivery tube was placed close to the nodule. In some experiments, the optical fiber probes of a nodule oximeter (Denison and Layzell, 1991) were placed against the nodule.

The nodule was viewed from beneath, through the plastic, using low-powered objective lenses, which were focused on the outermost circumference of the nodule. In early experiments, the reference electrode, filled with 2 M KCl in agar, was placed on the wet paper of the growth pouch at a remote distance from the nodule. The glass micropipette, filled with 2 M KCl, was brought into focus near the nodule on the side opposite the gas-delivery tube. The micropipette was then advanced into the nodule using a hydraulically controlled micropositioner. As the micropipette advanced, the E_m jumped between more and less negative values as seals were formed and broken between the micropipette and cell membranes. Once a suitable position was found (E_m more negative than about -80 mV), the E_m sometimes continued to become more negative for several minutes and then became stable, usually at values more negative than -100 mV. In later experiments, both the micropipette and the reference electrode were filled with 10 mM KCl, and the reference electrode was placed directly on the nodule.

Measurement of P_O

P_O was measured by nodule oximetry, as previously described (Denison and Layzell, 1991). Briefly, FOL was monitored in intact, attached nodules by fiber-optic spectrophotometry. A computer-controlled gas-mixing system bathed the attached nodule in 1 L/min of humidified gas, switching sequentially from air (20 kPa O_2 in N_2) to pure N_2, to 70 kPa O_2, and back to pure N_2. (Partial pressures assume a total atmospheric pressure of 100 kPa.) The 70 kPa O_2 exposure was continued only long enough for Lb to become O_2 saturated, typically <20 s. The central zone respiration rate was calculated from the rate of decrease in FOL after switching the gas stream surrounding the nodule from 70 kPa O_2 to pure N_2. P_O was calculated from the rate of increase in FOL after switching from pure N_2 to 70 kPa O_2, correcting for respiration. Calculated values of P_O and respiration rate from this procedure were based on an assumed Lb concentration of 0.68 mM (Bergersen, 1982). Actual Lb concentrations may vary within or among species, so changes in P_O with treatment will be emphasized rather than absolute values.

Gas Treatments

A brief (1–2 min) exposure of nodules to pure O_2 was used to induce a decrease in P_O. This short but relatively

Figure 1. Diagram of a pouch-grown soybean plant, showing root with attached nodule positioned for E_m and P_O measurements. The diagram illustrates the following items: 1, glass micropipette electrode showing its Ag/AgCl wire in contact with KCl solution; 2, reference electrode showing its Ag/AgCl wire and KCl-agar-filling material; 3, optic fibers leading to the nodule oximeter; 4, gas-delivery tube that also serves as the holder for the optic fibers; 5, plastic layers of the growth pouch; 6, paper layers of the growth pouch; 7, microscope stage; 8, objective lens; 9, condenser lens.
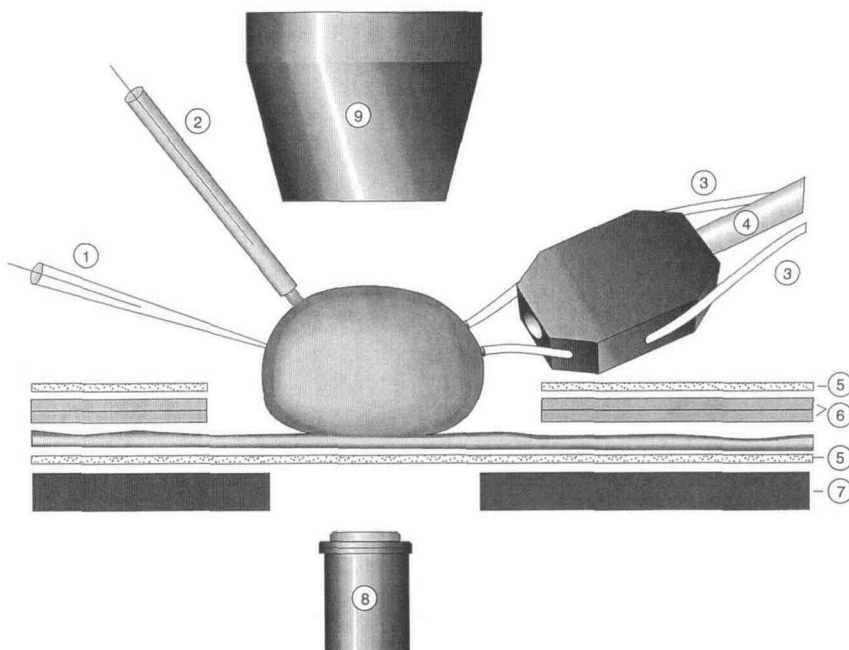
severe treatment was chosen because of the difficulty of maintaining microelectrode impalements for the times required for more gradual changes in O$_2$. In birdsfoot trefoil nodules, a gradual increase in external O$_2$ from 20 to 40 kPa during a 40-min period was previously found to cause a reversible decrease in P_O (Denison and Layzell, 1991). Such gradual changes do not appear to affect nitrogenase activity, at least in soybean (Hunt et al., 1989). Exposure of soybean nodules to pure O$_2$ for periods similar to those used in the current experiments caused a temporary interruption of nitrogenase activity (monitored as H$_2$ production), with 90% recovery typically in <20 min (Denison et al., 1992). Other gas treatments used in our experiments (e.g. brief exposure to pure N$_2$) will be discussed in context.

RESULTS

E_m Responses to O$_2$ Treatments

E_m values for nodules bathed in air (Fig. 2) were typical of those generally observed in nonphotosynthesizing cells. For all four species, the prolonged replacement of air by pure N$_2$ (i.e. 0 kPa O$_2$) nearly always caused a depolarization, but shorter exposure to anoxia sometimes failed to affect E_m. Depolarization induced by N$_2$ was readily reversed by a return to air, but occasionally an oscillation was seen in soybean nodules (Fig. 2). Oscillations in nitrogenase activity of soybean nodules have previously been reported with rapid increases in external O$_2$ concentration (Hunt et al., 1989).

For all four species, the replacement of air by pure O$_2$ caused depolarizations (Fig. 2), but the reliability of the response varied from species to species. For white clover and alfalfa, many strong responses were observed, and a failure to respond was very rare. Failure to respond was more common in soybean, but in the majority of cases, O$_2$ induced a depolarization, especially after we had improved our techniques for reducing disturbance of the plant and keeping the nodule moist. Only in the case of birdsfoot trefoil was the response unreliable.

The O$_2$-induced depolarization was reversed, sometimes after a delay, by a return to air. We often chased a brief exposure to O$_2$ with a brief exposure to N$_2$ (e.g. white clover and alfalfa, Fig. 2) to reduce O_i quickly, thus preventing irreversible injury and saving the nodule for further experimentation. If N$_2$ was maintained long enough (not shown), the membrane repolarized and then depolarized again, presumably because of a gradual decline in O_i to favorable levels followed by a further decline to levels insufficient to sustain respiration.

In contrast to the results obtained with nodules, measurements of E_m in uninoculated soybean and alfalfa roots showed that depolarization was induced by exposure to pure N$_2$ but not pure O$_2$ (results not shown).

Simultaneous Measurement of E_m and P_O

A series of experiments with soybean was undertaken to determine whether changes in P_O coincided with those in nodule E_m. Because P_O measurement by nodule oximetry
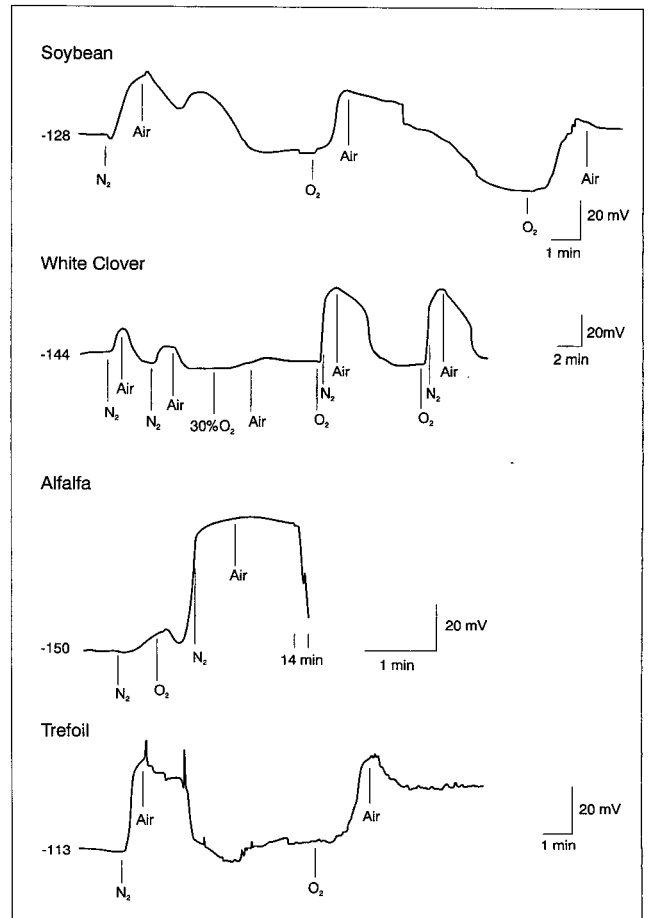

Figure 2. Chart records of E_m in legume nodules exposed to various atmospheres. The initial E_m (in mV) is recorded at the start of each trace, where air was the bathing atmosphere. Upward trends reflect decreasing negativity. Note that at the end of the alfalfa trace the chart speed was reduced for 14 min while waiting for the slow repolarization of the membrane. Some, probably artifactual, step changes in E_m generally occur during microelectrode measurements. These may reflect movements from vibrations or other sources.

requires 1 min or more for completion, it was impossible to measure exactly the time course of P_O changes relative to E_m changes. Figure 3 represents one of four experiments with soybean nodules. At approximately -11 min, the first oximeter assay for P_O was performed. The bathing gas was switched from air to N$_2$, then to 70 kPa O$_2$, then back to N$_2$, and finally to air. The resulting value for P_O (see "Materials and Methods") is written above the E_m trace. The oximeter assay was repeated about 5 min later (note oscillation in E_m during recovery). About 5 min after the second assay, at time zero, 100 kPa O$_2$ was administered for about 90 s until the E_m stabilized after the depolarization. The gas stream was then switched back to air. A few minutes later, after FOL (monitored by oximetry) had stabilized, but before the E_m had appreciably recovered, another P_O assay was performed. Two more oximeter assays were run after the recovery of E_m that occurred 6 to 8 min after the O$_2$ treatment.
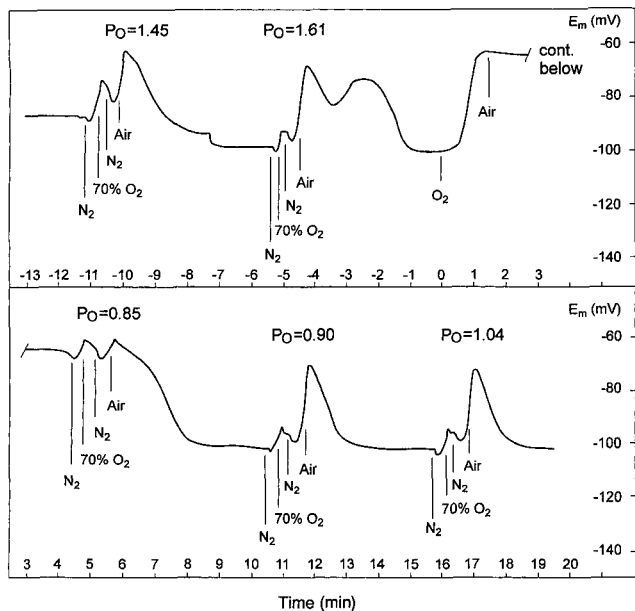
Figure 3. Chart record of one of four experiments in which E_m and P_O (in μm/s) were measured in soybean nodules. The oximeter assay for P_O requires the sequence of gas changes recorded early in the record. The same sequential gas changes were made four additional times. At time zero a 1- to 2-min O_2 pulse induced a depolarization lasting about 6 min.

In one of the experiments (not shown), seven nodule oximeter assays were run after the O_2 treatment, and the P_O continued to recover until the fifth assay (about 30 min). In another of the experiments, a second O_2 treatment was applied after the E_m recovered, which again induced a depolarization and a reduced P_O from which the nodule again recovered. In all four experiments, a fully depolarizing O_2 treatment was accompanied by a significant reduction in P_O, which subsequently increased without exception.
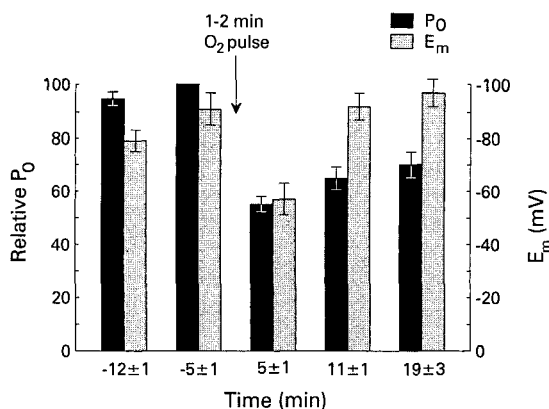
Figure 4 presents average values from the coordinated

Figure 4. Average values from coordinated E_m and P_O measurements in soybean nodules, based on four experiments similar to that shown in Figure 3. At time zero a 1- to 2-min pulse of O_2 was applied. P_O values are expressed relative to the value obtained just prior to the O_2 treatment.

E_m and P_O measurements on soybean. Values for P_O were normalized based on the oximeter assay done just prior to the exposure to O_2 to compensate for the variation in initial differences in apparent P_O among the nodules (values ranged from 0.76–1.61). Depolarization coincided with decreased P_O, within the time resolution of our approach, but P_O recovered more slowly than E_m.

DISCUSSION

Membrane depolarizations induced by anaerobiosis (e.g. under pure N_2) are common in aerobic organisms (Cheeseman and Hanson, 1979), but we have been unable to find any previous reports of depolarizations in higher plants caused by exposure to elevated O_2. There are at least three possible explanations for O_2-induced depolarizations in nodules, based on (a) O_2 inhibition of nitrogenase activity, (b) ion fluxes controlling P_O, or (c) signal transduction related to O_2 stress.

Nitrogenase activity was temporarily eliminated by similar O_2 treatments in a previous experiment with soybean (Denison et al., 1992). This cessation of N_2 fixation would be expected to substantially alter metabolism in the nodule central zone, but we are unable to suggest a simple way in which cessation of N_2 fixation would cause depolarization. Also, the rapid recovery in E_m that we observed contrasts with the gradual recovery in nitrogenase activity reported previously. If the recovery in E_m precedes the recovery in N_2 fixation, then the recovery in nitrogenase activity cannot be the cause of the repolarization.

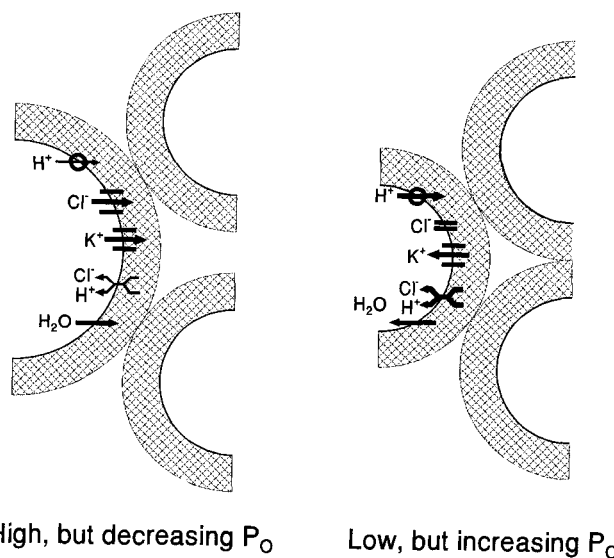An osmoelectrical explanation for O_2-induced depolarizations and their association with decreases in P_O is

Figure 5. A model for the osmoelectrical control of P_O. Essential components of the model include (a) a proton pump inhibited by elevated O_i; (b) a Cl^- channel that opens at elevated O_i; (c) a K^+-selective channel, not necessarily controlled by O_i; and (d) a co-transport system for Cl^- and H^+. The cross-hatched areas represent the water-filled apoplast external to the plasma membranes. Relative fluxes are indicated by the thickness of the arrows.

shown in Figure 5. Under this hypothesis, depolarizations are linked to fluxes of inorganic ions controlling turgor pressure in cells whose size and shape determine the dimensions of intercellular spaces roughly analogous to stomatal pores, thereby regulating nodule gas permeability. The ion fluxes required by the proposed mechanism are similar to those thought to occur in guard cells (Schroeder et al., 1993) and pulvinar motor cells (Lee, 1990).

Two groups of cells are shown in Figure 5, illustrating high but decreasing P_O (left) and low but increasing P_O (right). Under this hypothesis, a decrease in P_O would occur when high O_i caused a decrease in proton pump activity and the opening of an anion channel (via an unspecified signal transduction pathway), allowing efflux of Cl^-. The resulting depolarization would change the electrochemical gradient for K^+, causing efflux through a K^+ channel. This decrease in the cellular concentration of two major inorganic osmoticants would drive efflux of water, leading to decreased cell turgor. Recovery after removal of the O_2 stress (Fig. 5, right) would require increased proton pump activity, closing of the anion channel, and uptake of Cl^- by co-transport with protons. Repolarization would also drive uptake of K^+, and uptake of water would increase cell turgor. Depending on the capacity of the ion channels and co-transport system, recovery of turgor might be expected to lag behind recovery of E_m. The model makes the reasonable assumption that K^+ is ordinarily·near electrochemical equilibrium and that Cl^- is far from electrochemical equilibrium, with passive efflux of Cl^- favored irrespective of changes in E_m.

An essential feature of this osmoelectrical hypothesis is that efflux of water from cells causes a decrease in P_O. Figure 5 shows one way in which flux-induced changes in cell turgor might control P_O, without a requirement for highly differentiated cells. A single cell is postulated to expand and contract, increasing and decreasing the size of an intercellular space between the variable cell and two cells of fixed dimensions. We have not explored the biomechanical feasibility of this mechanism in any detail, but Figure 5 illustrates the idea that regulation of gas permeability by changes in cell turgor might not require the extreme anatomical specialization found in stomata. Recognizable stomata have not been reported in nodules, although they are found in many green stems and in some seeds and fruits (Jernstedt and Clark, 1979) as well as in leaves.

Typical nodule P_O values could be achieved by only a few tens of air-filled intercellular spaces per nodule through an otherwise continuous gas diffusion barrier (Denison, 1992). The fact that we observed depolarizations with O_2 exposure after most successful impalements (regardless of electrode tip location) indicates that the depolarizations are not limited to just a few cells per nodule. However, it should be noted that plant cells within a tissue are often electrically coupled (Spanswick, 1972). We accept the generally held view that primary control of P_O probably occurs externally to the nodule central zone. However, a similar mechanism could explain changes in the shape of intercellular spaces that can occur throughout the nodule interior (van Cauwenberghe et al., 1994).

The amount of change in P_O resulting from a given change in air-space dimensions would depend on whether the cell walls lining the space were hydrophobic, as in stomata, or hydrophilic. With hydrophilic walls, a decrease in the size of an intercellular space could cause it to fill, reversibly, with capillary water. This would drastically reduce flux of O_2 or other gases through that air space. Water efflux from symplast to apoplast could reduce P_O even without a change in air-space dimensions (Denison, 1992).

Denison et al. (1992) showed that H_2 concentrations inside soybean nodules are consistent with the presence of a small number of air-filled pathways through an aqueous diffusion barrier. More convincing experimental evidence for a gas-phase diffusion pathway into unstressed soybean nodules comes from the response of nodule respiration to atmospheres containing helium or argon (Witty and Minchin, 1994). The same authors also found that O_2 diffusion into stressed soybean nodules occurred primarily by liquid-phase diffusion. These results are consistent with opening and closing of air-filled pathways through an aqueous diffusion barrier, perhaps by a mechanism similar to that shown in Figure 5.

Although this is, to our knowledge, the first report of rapid and reversible changes in any nodule parameter that could plausibly cause rapid and reversible changes in P_O, the osmoelectrical hypothesis is clearly speculative at this point. The observed decline and recovery of P_O in nodules vacuum infiltrated with KCl (Purcell and Sinclair, 1994) are consistent with the postulated ability of nodule cells to take up the suggested inorganic ions, but their central role in regulation of P_O remains to be demonstrated. The fluxes of K^+ predicted by the osmoelectrical hypothesis should be measurable using either ion-specific microelectrodes or fluorescent probes, but identifying the cells involved could be difficult if they are few in number. Purcell and Sinclair (1994) recently suggested that fluxes of organic ions could also be associated with changes in nodule E_m.

An alternative explanation for O_2-induced depolarizations is that they form part of a signaling cascade. Depolarizations could trigger decreases in P_O even if the physical mechanism of P_O control does not directly involve ion fluxes. This hypothesis also seems plausible, given the known involvement of depolarization as a signal in the early stages of nodulation (Ehrhardt et al., 1992). Depolarizations may also be involved in plant responses to pathogens (Thain et al., 1990), which is particularly interesting given the involvement of an "oxidative burst" in plant defenses (Apostol et al., 1994). Under the signal cascade hypothesis, an increase in O_i might lead to the production of reactive O_2 species (e.g. hydrogen peroxide), which are thought to act as signal molecules (Bradley et al., 1992; Apostol et al., 1994). Depolarization might still involve fluxes of inorganic ions (e.g. of Ca^{2+}) but not necessarily in an osmotic role. The physical mechanism controlling P_O is not specified under the signal cascade hypothesis. Changes in the amount or hydrophobicity of glycoprotein in intercellular spaces (Witty and Minchin, 1990; James et al., 1991; Iannetta et al., 1993) would be one possibility. Whether or

not O_2-induced depolarizations prove to be directly connected to regulation of P_O, they appear to be unique to nodules and may provide further clues to the complex role of O_2 in nodule function.

If the osmoelectrical hypothesis is correct, then other treatments that cause a decrease in P_O (e.g. defoliation) should also cause at least some depolarization. The predictions of the signal cascade hypothesis would depend on whether depolarization is a general-purpose stress signal or only a signal of O_2 stress. The effects of gradual increases in O_2 would be of particular interest, but some modifications of the setup shown in Figure 1 would probably be needed to maintain longer microelectrode impalements.

LITERATURE CITED

Apostol I, Heinstein PF, Low PS (1994) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Role in defense and signal transduction. Plant Physiol 90: 109–116

Appleby CA (1984) Leghemoglobin and *Rhizobium* respiration. Annu Rev Plant Physiol 35: 443–478

Bergersen FJ (1982) Root Nodules of Legumes: Structure and Functions. Research Studies Press, Toronto, Canada

Bradley DJ, Kjellbom P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell 70: 21–30

Chaney RL, Bell PF (1987) Complexity of iron nutrition: lessons for plant-soil interaction research. J Plant Nutr 10: 963–994

Cheeseman JM, Hanson JB (1979) Energy-related potassium influx as related to cell potential in corn roots. Plant Physiol 64: 842–845

De Lorenzo C, Iannetta PPM, Fernandez-Pascual M, James EK, Lucas MM, Sprent JI, Witty JF, Minchin FR, de Felipe MR (1993) Oxygen diffusion in lupin nodules. II. Mechanisms of diffusion barrier operation. J Exp Bot 44: 1469–1474

Denison RF (1992) Mathematical modeling of oxygen diffusion and respiration in legume root nodules. Plant Physiol 98: 901–907

Denison RF, Layzell DB (1991) Measurement of legume nodule respiration and O_2 permeability by noninvasive spectrophotometry of leghemoglobin. Plant Physiol 96: 137–143

Denison RF, Witty JF, Minchin FR (1992) Reversible O_2 inhibition of nitrogenase activity in attached soybean nodules. Plant Physiol 100: 1863–1868

Ehrhardt DW, Atkinson EM, Long SR (1992) Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science 256: 998-1000

Hartwig U, Boller B, Nösberger J (1987) Oxygen supply limits nitrogenase activity of clover nodules after defoliation. Ann Bot 59: 285–291

Hunt S, King BJ, Layzell DB (1989) Effects of gradual increases in O_2 concentration on nodule activity in soybean. Plant Physiol 91: 315–321

Hunt S, Layzell DB (1993) Gas exchange of legume nodules and the regulation of nitrogenase activity. Annu Rev Plant Physiol Plant Mol Biol 44: 483–511

Iannetta PPM, De Lorenzo C, James EK, Fernandez-Pascual M,

Sprent JI, Lucas MM, Witty JF, de Felipe MR, Minchin FR (1993) Oxygen diffusion in lupin nodules. I. Visualization of diffusion barrier operation. J Exp Bot 44: 1461–1467

James EK, Sprent JI, Minchin FR, Brewin NJ (1991) Intercellular location of glycoprotein in soybean nodules: effect of altered rhizosphere oxygen concentration. Plant Cell Environ 14: 467–476

Jernstedt JA, Clark C (1979) Stomata on the fruits and seeds of Escholzia (Papaveraceae). Am J Bot 66: 586–590

King BJ, Hunt S, Weagle GE, Walsh KB, Pottier RH, Canvin DT, Layzell DB (1988) Regulation of O_2 concentration in soybean nodules observed by *in situ* spectroscopic measurement of leghemoglobin oxygenation. Plant Physiol 87: 296–299

Layzell DB, Hunt S, Moloney AHM, Fernando SM, Diaz del Castillo L (1990) Physiological, metabolic and developmental implications of O_2 regulation in legume nodules. *In* PM Gresshoff, LE Roth, G Stacey, WE Newton, eds, Nitrogen Fixation: Achievements and Objectives. Chapman and Hall, New York, pp 21–32

Lee Y (1990) Ion movements that control pulvinar curvature in nyctinastic legumes. *In* RL Satter, HL Gorton TC Vogelmann, eds, The Pulvinus: Motor Organ for Leaf Movement. American Society of Plant Physiologists, Rockville, MD, pp 130–141

Purcell LC, Sinclair TR (1994) An osmotic hypothesis for the regulation of oxygen permeability in soybean nodules. Plant Cell Environ 17: 837–843

Schroeder JI, Schmidt C, Sheaffer J (1993) Identification of high-affinity slow anion channel blockers and evidence for stomatal regulation by slow anion channels in guard cells. Plant Cell 5: 1831–1841

Schuller KA, Minchin FR, Gresshoff PM (1988) Nitrogenase activity and oxygen diffusion in nodules of soyabean cv. Bragg and a supernodulating mutant: effects of nitrate. J Exp Bot 39: 865–877

Spanswick RM (1972) Electrical coupling between the cells of higher plants: a direct demonstration of intercellular transport. Planta 102: 215–227

Streeter JG (1992) Analysis of apoplastic solutes in the cortex of soybean nodules. Physiol Plant 84: 584–592

Thain JF, Doherty HM, Bowles DJ, Wildon DC (1990) Oligosaccharides that induce proteinase inhibitor activity in tomato plants cause depolarization of tomato leaf cells. Plant Cell Environ 13: 569–574

Tjepkema JD, Yocum CS (1974) Measurement of oxygen partial pressure within soybean nodules by oxygen microelectrodes. Planta 119: 351–360

van Cauwenberghe OR, Hunt S, Newcomb W, Canny MJ, Layzell DB (1994) Evidence that short-term regulation of nodule permeability does not occur in the inner cortex. Physiol Plant 91: 477–487

Vessey JK, Layzell DB (1987) Regulation of assimilate partitioning in soybean. Initial effects following change in nitrate supply. Plant Physiol 83: 341–348

Weisz PR, Denison RF, Sinclair TR (1985) Response to drought stress of nitrogen fixation (acetylene reduction) rates by field-grown soybeans. Plant Physiol 78: 525–530

Witty JF, Minchin FR (1990) Oxygen diffusion in the legume root nodule. *In* PM Gresshoff, LE Roth, G Stacey WE Newton, eds, Nitrogen Fixation: Achievements and Objectives. Chapman and Hall, New York, pp 285–292

Witty JF, Minchin FR (1994) A new method to detect the presence of continuous gas-filled pathways for oxygen diffusion in legume nodules. J Exp Bot 45: 967–978